

Bovine Mastitis Associated with *Prototheca blaschkeae*[▽]

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Bovine mastitis is an important and complex disease responsible for economic losses in the dairy industry. Biotype II strains of the green alga *Prototheca zopfii* can be involved, most often resulting in chronic mastitis of difficult treatment associated with reduced milk production. This type of infection is rare, but the number of reported cases is increasing worldwide. In order to determine the kind of species involved in mastitis by *Prototheca* in northwest Portugal, 41 *Prototheca* isolates were genetically characterized. The algae are part of *Prototheca* isolates that were collected during a 6-year period, isolated from the milk of 41 dairy cows in a total of 22 herds with a history of increasing somatic cell counts, mild clinical signs of udder infection, and unsuccessful response to the usual therapy. PCR amplification of the 18S ribosomal DNA (rDNA), amplified rDNA restriction analysis, and phylogenetic analyses of the 18S rDNA sequences were performed. Thirty-seven isolates were identified as *P. zopfii* var. *hydrocarborea* and four as *Prototheca blaschkeae*. These data suggest a high incidence of *P. zopfii* var. *hydrocarborea* mastitis in the region and demonstrate for the first time the involvement of *P. blaschkeae* with bovine mammary gland infections.

Green algae of the genus *Prototheca*, and more rarely *Chlorella*, are the only known plant-like organisms that cause infectious diseases in humans and animals (6, 11–13). The genus *Prototheca* consists of microscopical, unicellular, achlorophyllic algae with asexual reproduction by autosporeulation with variable numbers of autospores (5, 10). These algae are ubiquitous and saprophytic, but some species may turn into unusual opportunists causing pathology when the host immunological defenses are impaired (16, 18, 21) or when predisposing factors occur, such as, in the case of dairy cows, poor animal care and poor milking hygiene (8, 11, 18). The incidence of bovine mastitis associated with *Prototheca* infections is steadily increasing and gaining more and more economic and public health importance (18). *Prototheca* is included in the class Trebouxiophyceae (14) and is closely related to green algae of the genus *Chlorella* (13, 23), which are among the best-studied unicellular green algae (7). Within the last decade, *Prototheca* taxonomy has developed and five species are currently assigned to the genus, namely *Prototheca zopfii*, *P. wickerhamii*, *P. stagnora*, *P. ulmea*, and most recently, *P. blaschkeae* (19). The validation of another species, *Prototheca moriformis* (1, 17), has been questioned by molecular studies (19, 25). Until recently, the only species known to cause infectious diseases in humans and animals were *P. wickerhamii* and *P. zopfii*, respectively (4, 20, 28). The new species *P. blaschkeae* was identified in 2006 from a previous case of human onychomycosis (19). This spe-

cies was previously defined as biotype III of *P. zopfii*, which, together with biotype I, was reported to be nonpathogenic, while biotype II has been associated with bovine mastitis (20). Biotype classification was based on phenotypic characteristics, as well as auxonographical and biochemical analyses (3, 20). Serological analyses revealed differences in the pattern of immunogenic structures between the biotypes (20). Further phylogenetic studies of 18S ribosomal DNA (rDNA) sequences were able to determine discriminatory molecular characteristics to define the new species *P. blaschkeae* and reclassify the *P. zopfii* biotypes into two genotypes (19).

The present study aimed to elucidate the epidemiology of bovine mastitis by molecular characterization of *Prototheca* isolates obtained from 41 dairy cows of 22 herds in Portugal. We report the predominant occurrence of *Prototheca zopfii* var. *hydrocarborea* in milk of cows with mastitis associated with *Prototheca* and for the first time the association of *P. blaschkeae* with bovine mastitis.

MATERIALS AND METHODS

Isolation of algae. *Prototheca* isolates in this study belong to a major collection compiling several milk pathogens that belong to the Laboratory of Infectious Diseases of the Veterinary Medicine School of Porto University. These represent a small number of pathogenic organisms that were recovered from about 3,500 mastitic milk analyses during a 6-year period (from January 2002 to December 2007). *Prototheca* cells were retrieved from milk of 41 cows with mastitis originating from 22 different dairy herds from northwest Portugal, which was collected under sterile conditions. The affected cows had a history of persistently high somatic cell counts or clinical mastitis that was not responsive to intramammary antibiotic treatment. To avoid confounding effects due to the therapy, only milk samples collected after the treatment safety period were analyzed. For diagnostic purposes, 40-μl aliquots of milk samples collected from individual quarters of the udder were streaked onto Columbia agar plates supplemented with 5% sheep blood (bioMérieux, Marcy l'Etoile, France). After 42 to 72 h at

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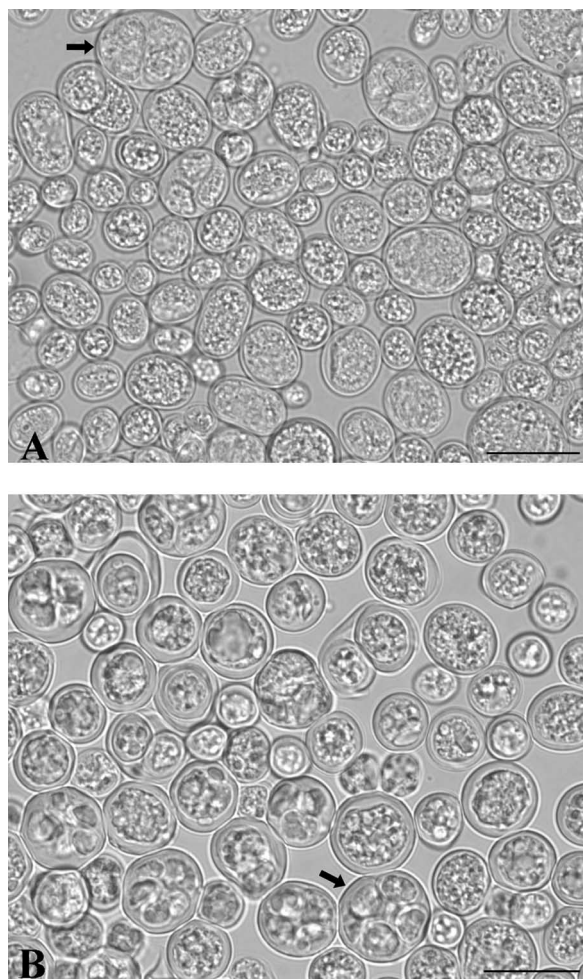


FIG. 1. Photomicrographs obtained by light microscopy of wet mount preparations of *Prototheca* spp. stained with lactophenol cotton blue. (A) Characteristic morphology of *P. zopfii* sporangiospores within sporangia (arrow). (B) Regularly organized sporangiospores of *P. blaschkeae* within the sporangia (arrow). Magnification, $\times 1,000$. Bar, 20 μm .

37°C, plates were examined for *Prototheca* growth and any colonies resembling this alga were subcultured once on Sabouraud dextrose agar medium (Merck Laboratories, Darmstadt, Germany). After macroscopical and microscopical identification, *Prototheca* isolates were spread and grown on Sabouraud dextrose agar medium.

Preparation of genomic DNA. Algal cultures were grown on Sabouraud dextrose agar for 48 h at 37°C. Cells were harvested by centrifugation ($1,600 \times g$, 10 min) and mechanically ground with glass beads and extraction buffer (200 mM Tris-HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate) in 1.5-ml Eppendorf tubes in a cell mill (Qiagen tissue lyser) for 10 min at 30 Hz. The efficiency of cell disruption was controlled by microscopical observation. Subsequently, 200 μl of chloroform was added and the suspension was shaken for 1 min. After centrifugation ($21,000 \times g$, 5 min), the supernatant was transferred to a new tube and mixed with 200 μl of isopropanol for DNA precipitation. The DNA was pelleted by centrifugation ($21,000 \times g$, 5 min) and washed with 70% ethanol. The pellet was air dried and resuspended in a maximum of 50 μl of Milli-Q sterilized water for further use.

rDNA amplification and amplified rDNA restriction analysis. For amplification of the 18S rDNA, the conserved eukaryote-specific primers previously designed by Huss et al. (7) (forward primer, 5' WACCTGGTTGATCCTGCCAGT 3'; reverse primer, 5' GATCCTTCYGCAGGTTACCTAC 3') were used. Thirty-five cycles were run in a Biometra T3000 thermal cycler with 60 s of denaturation at 95°C, 60 s of annealing at 54°C, and 150 s of extension at 72°C. The amplification products were analyzed on a 0.8% (wt/vol) agarose gel after

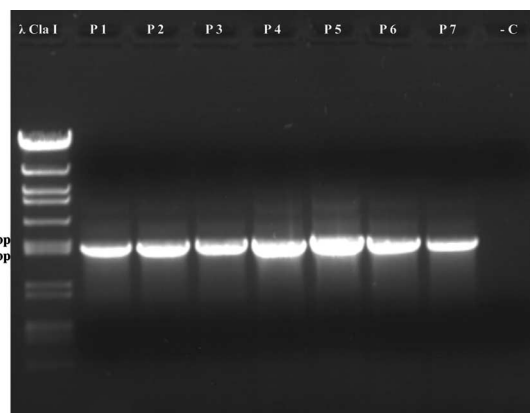


FIG. 2. 18S rDNA amplification of some *Prototheca* isolates (P1 to P7) with eukaryote-specific PCR amplification primers. All PCR fragments have a size of approximately 1,800 bp. Lane λ ClaI, molecular weight standard; lane -C, negative control without DNA.

staining with ethidium bromide. The amplified rDNA was submitted to restriction analysis with HaeIII (NEB, Inc., Frankfurt, Germany) and analyzed on a 1.8% (wt/vol) agarose gel after staining with ethidium bromide. Several of the amplified rDNAs were purified with a PCR purification kit (Qiagen, Inc., Hilden, Germany) and directly sequenced using the following primers: 300 F (5' AGG GTTCGATTCCGGAG 3'), 1055 F (5' GGTGGTGCATGGCCG 3'), 536 R (5' GWATTACCGCGGCKGCTG 3'), and 1200 R (5' GGGCATCACAGACCTG 3') (7). Nucleotide sequence determination was carried out by MacroGen, Ltd. (Korea).

Phylogenetic analyses. The 18S rDNA sequences determined in this study were manually aligned on a MicroVAX computer with the sequence editor program distributed by G. Olsen (15). For the phylogenetic analyses, the sequences were aligned with sequences of representative trebouxiophycean green algae extracted from a larger alignment and with two chlorophycean algae that were used as an outgroup. To improve the alignment of the data set, secondary structure models were constructed according to the model provided by Wuyts et al. (27). Highly variable regions that could not be aligned unambiguously for all sequences were excluded from the analyses together with PCR primer binding regions, resulting in a total of 1,782 positions. Phylogenetic trees were inferred from the aligned sequence data by the neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) method using PAUP* 4.0 (22). One

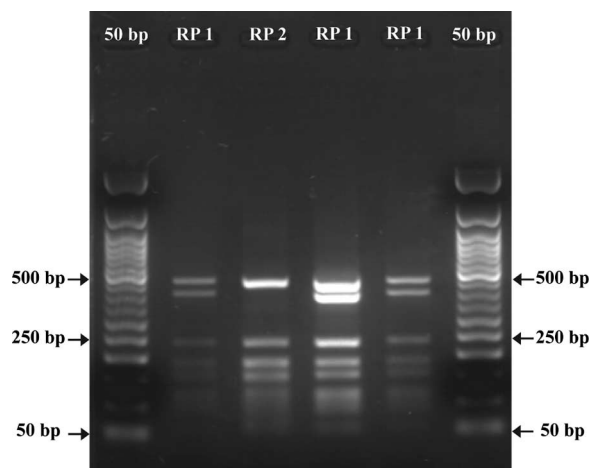


FIG. 3. Restriction patterns of amplified 18S rDNA fragments of four *Prototheca* isolates. PCR fragments were digested with HaeIII, yielding two different patterns, RP 1 and RP 2. RP 2 lacks the 400-bp fragment observed in RP 1. Lanes 50 bp, molecular weight standard (50-bp ladder).

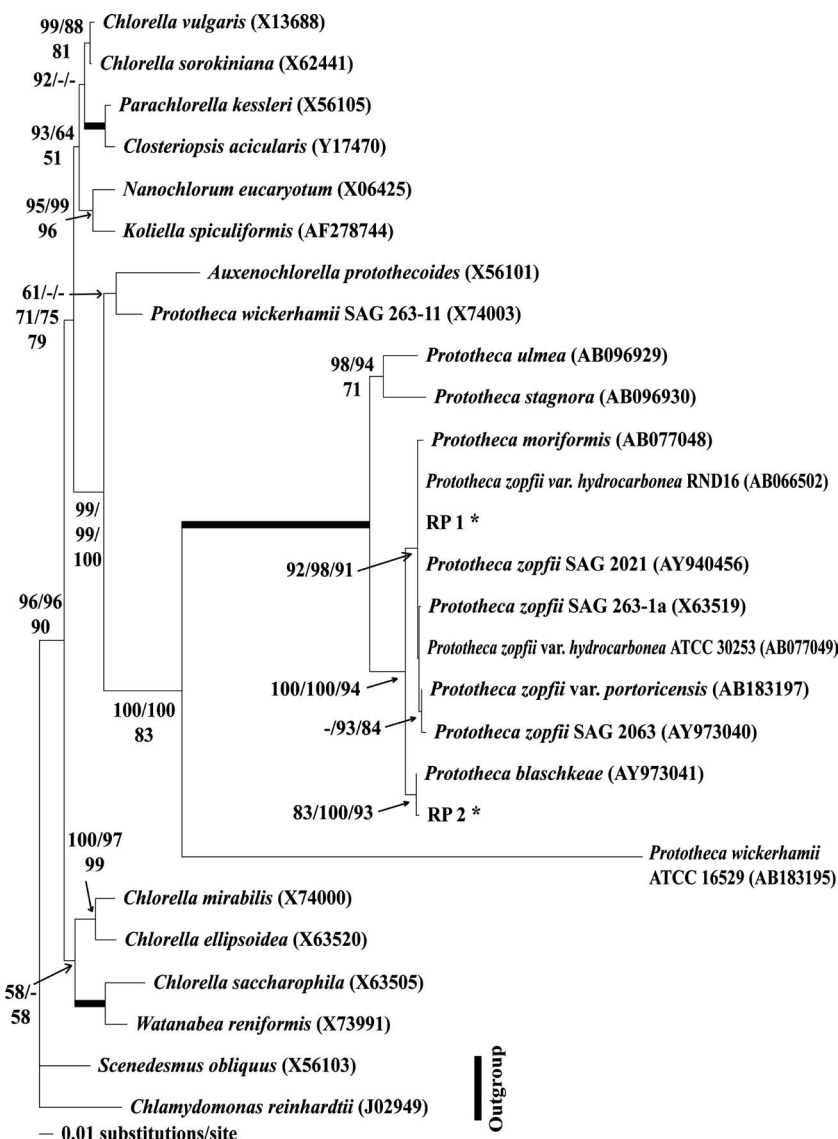


FIG. 4. Phylogenetic tree inferred from complete or almost complete 18S rRNA gene sequences of trebouxiophycean green algae showing species affiliation of *Prototheca* isolates representing RP 1 and RP 2 (see Fig. 3). The tree topology is derived from an ML analysis. Bootstrap values are shown at the internal nodes for each 1,000 resamplings of NJ and MP and 100 resamplings for ML, if values are higher than 50%. Thick lines indicate 100% bootstrap support for all three methods. Asterisks indicate sequences determined in this study. Branch lengths correspond to evolutionary distances. Bar, 0.01 nucleotide substitution per site.

thousand bootstrap replicates each were carried out for NJ and MP, as well as 100 replicates for ML.

Nucleotide sequence accession numbers. The 18S rRNA gene sequences determined in this study have been deposited in GenBank under accession no. EU439262 and EU439263.

RESULTS

The recovery of the 41 *Prototheca* isolates from mastitic milk from 41 different dairy cows was achieved by usual microbiological methods. After milk samples were streaked on Columbia agar supplemented with 5% sheep blood or on Sabouraud dextrose agar and incubated for 48 to 72 h, grayish and white to cream-colored small colonies of 1 mm or 1 to 2 mm, respectively, with yeast-like appearance and odor were detected. No hemolysis was observed on the Columbia sheep blood agar

plates. Four of the *Prototheca* isolates, obtained from cows of different herds, showed slower growth and paler and smoother colonies. The microscopical observation of *Prototheca* cells stained with lactophenol cotton blue showed a typical appearance with ovoid to globose sporangia with sporangiospores in several developmental stages (Fig. 1A). However, some differences in the internal organizations of sporangiospores of the four isolates mentioned above were observed. Their sporangiospores were more consistently and regularly organized than those of the other isolates (Fig. 1B), suggesting that they could belong to a different *Prototheca* species. The morphological appearance observed for the *Prototheca* spp. was in concordance with the previous description of Pore (16) and DiPersio (5) for *P. zopfii* and of Roesler et al. (19) for *P. blaschkeae*.

To determine the species of *Prototheca* associated with bovine mastitis in this study, the 18S rRNA genes of all isolates were amplified (Fig. 2). For each isolate analyzed, a PCR fragment of about 1,800 bp was detected as expected, since the sequence length of *P. zopfii* 18S rDNA is about this size (24). Each amplified rDNA was submitted to restriction analysis with HaeIII, resulting in two different restriction patterns (Fig. 3). For 37 isolates, a restriction pattern defined as restriction pattern 1 (RP 1) was identified. The four morphologically distinct isolates showed a different restriction pattern referred to as RP 2, which could be clearly distinguished from RP 1 by the lack of a 400-bp fragment (Fig. 3). The 18S rRNA genes from eight isolates representing restriction pattern RP 1 and four with RP 2 were completely sequenced. All sequences with RP 1 had a length of 1,807 bp and were identical to each other and also to the published sequence of *Prototheca zopfii* var. *hydrocarborea* strain RND16 (GenBank accession no. AB066502). The four isolates displaying RP 2 were also identical among themselves, had a length of 1,815 bp, and showed 99.8% identity (with three differences) within the 1,449 bp available for the 18S rDNA sequence of *P. blaschkeae* SAG 2064 (AY973041). The three differences were found in variable regions of the 18S rRNA. Similarity between the two types of sequences that displayed different restriction patterns was only 98.0%, corresponding to 40 differences including indels (insertions/deletions). Phylogenetic analyses with the NJ, MP, and ML method confirmed that most of our isolates belong to the *P. zopfii* clade, specifically to *P. zopfii* var. *hydrocarborea* and *P. zopfii* SAG 2021, while there is high bootstrap support that four isolates are more closely related to *P. blaschkeae* (Fig. 4).

DISCUSSION

Prototheca species are known to be widely dispersed within dairy farmlands. The occurrence of mastitis due to these algae usually takes place during periods of warm weather with high rainfall (8, 12, 16), reflecting poor management and hygiene combined with insufficient premilking cleaning and disinfection of the teats. Generally, infections are maintained in a herd by clinically healthy shedders (8, 18). The objective of this work was to determine the species of *Prototheca* that are associated with bovine mastitis in the northwest of Portugal. The results of morphological characterization and of the molecular analyses of the 18S rRNA genes demonstrated that most of our isolates, with RP 1, belong to biotype II of *P. zopfii* represented by strain SAG 2021 in Fig. 4, as suggested by Roesler et al. (20). This was not unexpected, as all algae associated with bovine mastitis have been identified as *P. zopfii* biotype II (2, 13, 18) and are generally considered to be its causative agent (13). Surprisingly, these isolates share complete 18S rDNA sequence identity with *P. zopfii* var. *hydrocarborea* RND16, a variety of *P. zopfii* which was first described in 2002 by Ueno et al. (26). This thermotolerant strain was isolated from a hot spring and was never associated with bovine mastitis. It would be interesting to know if this strain is potentially infectious or not. Differences in infectivity combined with the knowledge of characteristic physiological differences could be used to specify prerequisites for the pathogenicity of biotype II strains. In this context, determination of fermentation patterns of our isolates

might reveal essential differences, as heterogeneities for strains of a single species of *P. zopfii* are well known (9).

In a recent study (19), biotype I and II strains were reclassified as two genotypes of *P. zopfii* by 18S rRNA gene sequence analysis and determination of cellular fatty acids, and biotype III strains were defined as a new species, *P. blaschkeae*. Interestingly, four of our isolates, those with RP 2, share much more sequence similarity with *P. blaschkeae* than with *P. zopfii* (99.8% compared to 98%). As *P. blaschkeae* so far had been isolated only from a case of human onychomycosis (19), the results of our study demonstrate for the first time the involvement of *P. blaschkeae* in the etiology of bovine mastitis. To quickly discern between the two species, we also want to point out the important additional information provided by amplified rDNA restriction analysis, which allows fast screening of large numbers of isolates for the detection of different infectious species.

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